

Published in final edited form as:

Gen Comp Endocrinol. 2016 January 15; 226: 5–13. doi:10.1016/j.ygcen.2015.12.006.

Urinary iodine and stable isotope analysis to examine habitat influences on thyroid hormones among coastal dwelling American alligators

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Abstract

The American alligator, generally a freshwater species, is known to forage in marine environments despite the lack of a salt secreting gland found in other crocodylids. Estuarine and marine foraging could lead to increased dietary uptake of iodine, a nutrient necessary for the production of thyroid hormones. To explore the influence of dietary iodine on thyroid hormone health of coastal dwelling alligators, we described the seasonal plasma thyroxine and triiodothyronine concentrations measured by radioimmunoassay and urinary iodine (UI) concentrations measured by inductively coupled plasma mass spectrometry. We also analyzed long-term dietary patterns through stable isotope analysis of scute tissue. Snout-to-vent length (SVL) was a significant factor among UI and stable isotope analyses. Large adult males greater than 135 cm SVL had the highest UI concentrations but did not display seasonality of thyroid hormones. Alligators under 135 SVL exhibited seasonality in thyroid hormones and a positive relationship between UI and triiodothyronine concentrations. Isotopic signatures provided supporting evidence that large males predominantly feed on marine/estuarine prey whereas females showed reliance on freshwater/terrestrial prey supplemented by marine/estuarine prey. UI measurement provided immediate information that correlated to thyroid hormone concentrations whereas stable isotope analysis described long-term dietary patterns. Both techniques demonstrate that adult alligators in coastal

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environments are utilizing estuarine/marine habitats, which could alter thyroid hormone physiology.

Graphical Abstract



Keywords

American alligator; thyroid hormone; thyroxine; triiodothyronine; urinary iodine; stable isotope analysis

1. Introduction

Alligatorids including American alligators (*Alligator mississippiensis*) are distinct from closely related crocodylids in that they do not have lingual salt secreting glands, thereby limiting their use of marine and estuarine habitats (Taplin et al., 1982). However, alligators will forage in marine and estuarine habitats, provided there is a reliable nearby source of low salinity water (Nifong et al., 2015; Nifong et al., 2014; Rosenblatt and Heithaus, 2011; Rosenblatt et al., 2013). The iodine content of estuaries has been shown to be dependent on the marine endmember sources, with more iodine in the saltier environments (Cook et al., 2000; Truesdale and Upstill-Goddard, 2003). Additionally, marine prey contain more iodine than freshwater prey (Eckhoff and Maage, 1997). Therefore, coastal environments provide an iodine-rich diet compared to freshwater environments. Iodine is a dietary nutrient required for the production of the thyroid hormones thyroxine and triiodothyronine (T_4 and T_3 , respectively), which contribute to regulation of growth, metabolism, and reproduction (Flamant and Samarut, 2003; Trokoudes et al., 2006; Umpleby and Russell-Jones, 1996). A diet overly rich in iodine can induce hypothyroidism or hyperthyroidism in some species (Markou et al., 2001; Martin et al., 2000; Stanbury et al., 1998) and has been linked to hyperthyroid biomarkers in neonatal American alligators (Boggs et al., 2013). It thereby follows that because iodine is elevated in saltwater and marine prey compared to freshwater prey items and iodine is a nutrient required for production of thyroid hormones, alligators in coastal regions could have altered thyroid hormone concentrations due to an increased exposure to environmental iodine when foraging on marine/estuarine prey.

Merritt Island National Wildlife Refuge (MINWR) in Florida is located on a barrier island and houses the facilities for the National Aeronautics and Space Administration's (NASA) Kennedy Space Center (KSC). Although MINWR consists primarily of estuarine and marine ecosystems, it supports a robust alligator population. Activities at MINWR such as impoundment regulation for mosquito control and bird migrations, and retention ponds for KSC activities have created a variety of freshwater resources that potentially contribute to use of high salinity environments by the resident alligator population. Moreover, previous research at MINWR demonstrates alligators travel to and forage within associated marine and estuarine environments (Nifong et al., 2014). However, intra-population variation in these cross-ecosystem foraging behaviors has yet to be determined and can potentially have significant impacts on alligator health due to the effects of increased iodine on thyroid hormone synthesis.

Previous research has shown that neonatal alligators from saline habitats at MINWR exhibit a strong correlation between plasma inorganic iodide and the biologically active thyroid hormone, T_3 , which was not observed in the two freshwater populations from the same study (Boggs et al., 2013). This relationship is not present in juvenile alligators from MINWR, as there was no correlation between plasma inorganic iodide and thyroid hormones despite a strong correlations between plasma inorganic iodide and thyroid hormones among the neonatal population (Boggs et al., 2011). However, plasma inorganic iodine is a marker of circulating iodine, not iodine intake. It is possible that the larger/older alligators at MINWR may be regulating plasma iodide by increasing urinary iodine excretion, but this has not been explored previously.

Therefore, in this study, we describe the thyroid hormone seasonal cycles of sexually mature adult alligators at MINWR and examine urinary iodine (UI) concentrations to explore if excretion of excess iodine is correlated to thyroid hormone concentrations among different size classes and sexes of adult alligators inhabiting MINWR. We then use stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) to estimate the proportional contributions of marine/estuarine and freshwater/terrestrial prey to the diet of juvenile and adult alligators. Combined, these analyses provide a detailed assessment of intra-population variation in marine foraging and the potential implications of these behaviors for alligator endocrinology and health.

2. Materials and Methods

2.1 Animals and Tissue/Fluid Sampling

All animal care and use was performed in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC) under Protocol No. 201005071 and IACUC GRD-06-044. Samples were collected under MINWR permit 2006 SUP 55 and permits from the Florida Fish and Wildlife Conservation Commission and U.S. Fish and Wildlife Service.

Blood sampling and morphometrics were collected according to Myburgh et al. (2014) and specifics for this collection can be found in Hamlin et al. (2011). Briefly, all animals (2006 to 2010; $n = 595$) were actively captured at MINWR and KSC and an immediate blood sample taken upon restraint. Blood was collected in a lithium heparin Vacutainer (BD

367884), kept on ice until returned to the laboratory, and centrifuged to obtain plasma. Aliquots were frozen at -20°C .

Urine samples ($n = 174$) were obtained from 2007 to 2010 using the method described by Myburgh et al. (2012). Briefly, urine was collected in 50 mL Falcon tubes using a canine urinary catheter inserted into the urodeum via the cloaca. Urine was kept on ice until returned to the laboratory and then frozen at -20°C .

From 2006 to 2013 a single caudal scute (superficial scale-like structures of the integument; $n = 314$) was removed from captured individuals using a sterile scalpel, and used for stable isotope analysis. Scute samples were placed on ice in the field and immediately frozen at -20°C in the laboratory until further processing. After cleaning with deionized water to remove foreign debris, the keratinous epidermal layer was separated from dermal collagen layer of the scute using NaOH digestion (Alibardi and Thompson, 2000; Radloff et al., 2012). Isolated keratin samples were dried for 48 hours at 60°C and ground to fine powder. *Alligator mississippiensis* scute tissue has a slow rate of turnover (mean turnover = 590 days for $\delta^{13}\text{C}$ and 414 days for $\delta^{15}\text{N}$) (Rosenblatt and Heithaus, 2013) thus stable isotope signatures of scute keratin represent incorporation of long-term dietary patterns (> 1 year).

In 2013, samples of representative alligator prey species from both marine/estuarine and freshwater habitats within MINWR were collected to calculate end member values for use in our isotopic mixing model analysis. Prey item isotope samples were collected by the Florida Fish and Wildlife Conservation Commission – Fish and Wildlife Research Institute's Fisheries-Independent Monitoring Program. Collection and processing of tissue samples followed methods described in Nifong et al. (2015).

2.2 UI Measurements

Two bottles each of National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 3668 Frozen Human Urine, Level 1 and Level 2 having a certified value of $142.7 \mu\text{g/L} \pm 1.6 \mu\text{g/L}$ and $279.0 \mu\text{g/L} \pm 3.9 \mu\text{g/L}$, respectively, were used as control materials for iodine measurements.

Urine samples were agitated before approximately 0.5 mL was pipetted into a 15 mL polypropylene centrifuge tube followed by the addition of 5 mmol/L sodium hydroxide volumetrically diluting up to 10 mL. Samples resulting in high iodine concentrations, beyond the calibration curve, were further diluted by half and analyzed. In addition to the samples and control materials, twenty-one process blanks were carried through the entire sample processing and measurement scheme. The average blank mass fraction of iodine was $0.12 \mu\text{g/L}$, corresponding to a blank contribution of 0.08 % of the total iodine measured in SRM 3668 and was used to correct the sample measurement data.

All samples were measured by monitoring ^{127}I using inductively coupled plasma mass spectrometry (ICP-MS) on a Thermo X7 system (software build, 2.3.0.161) operating in regular mode. The ICP-MS was tuned and optimized using a standard 1 ng/g multi-element tuning solution. Quadrupole MS routine methods utilize peak jumping, with each of five replicate runs consisting of 150 sweeps (10 s acquisition time), and a dwell time of 30 ms.

The working ^{127}I calibration stock solution was prepared by gravimetric dilution of a high-purity primary standard (SRM 3180, Iodide Anion (I^-) Standard Solution). External calibration curves were constructed using SRM 3180 dilutions. A first order linear fit was applied to the data. The slope and intercept that form the calibration curve was based on the measured ^{127}I response from SRM 3180, and utilized to calculate the mass fraction of iodine in the urine samples.

UI concentrations were not corrected by creatinine for several reasons. The nephritic system of alligators lacks a loop of henle found in mammals, and unlike the saltwater crocodile (*Crocodylus porosus*), there is little osmotic regulation by the cloaca of American alligators exposed to saltwater (Moore et al., 2009; Pidcock et al., 1997). A subsample of alligator urine from this study was tested for creatinine using ELISA techniques (Cayman Chemical 500701). Concentrations of creatinine in alligator urine were below the detection limits of the kit, most likely due to the inability of alligators to concentrate urine. Additionally, in this study, iodine concentration is hypothesized to be directly related to saltwater exposure. Therefore normalizing by a factor which changes in response to osmotic challenges such as saltwater would bias results. For these reasons, creatinine was not used as a normalization factor for UI.

2.3 Stable Isotope Analysis

Stable isotope analysis is increasingly used by ecologists to elucidate patterns in food web structure and habitat use (Layman et al., 2012). This is due to the fact that the isotopic composition of a consumer's tissues closely tracks the isotopic composition of their diet once adjusted for any isotopic changes due to metabolism and tissue synthesis (DeNiro and Epstein, 1978; Peterson and Fry, 1987). The ratio of ^{15}N to ^{14}N (expressed relative to a standard as $\delta^{15}\text{N}$) is most often used to infer trophic position due to the step-wise enrichment of ^{15}N in consumer tissues along a food chain (Post, 2002). The ratio of ^{13}C to ^{12}C is primarily used to indicate use of particular carbon resources, since $\delta^{13}\text{C}$ varies substantially across primary producers differing in photosynthetic pathways (C_3 versus C_4) (O'Leary, 1981) and changes relatively little during trophic transfers ($1\text{‰} \pm 1\text{ SD}$) (Peterson and Fry, 1987).

For isotopic analysis, approximately 500 μg to 800 μg of homogenized animal (prey item) tissues or 1 mg to 3 mg of plant tissues was weighed and loaded into 9 mm \times 5 mm tin capsules at the University of Florida Geology Stable Isotope Laboratory, Gainesville, Florida. Analyses were performed using one of two systems: either a Finnigan DeltaPlus XL isotope mass spectrometer with ConFlo III interface linked to a Costech ECS 4010 Elemental Combustion System (elemental analyzer) or Finnigan-MAT 252 isotope ratio mass spectrometer coupled with a ConFlo II interface linked to a Carlo Erba NA 1500 CNS Elemental Analyzer. Stable isotope values are expressed in standard per mil notation (‰):

$$\delta X(\text{‰}) = [R_{\text{sample}}/R_{\text{standard}} - 1] \times 1000$$

where X is the element of interest and R is the ratio of heavy to light isotopes ($^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$) of the sample and standard (Vienna Pee Dee Belemnite used for $\delta^{13}\text{C}$ and

Atmospheric Nitrogen-AIR for $\delta^{15}\text{N}$). Instrument accuracy was measured and corrected for during each sample run, using four measures of in-laboratory standard USGS-40 (l-glutamic acid, $\delta^{13}\text{C} = -26.39$ and $\delta^{15}\text{N} = -4.52$).

2.4 Thyroid Hormone Measurements

Radioimmunoassays (RIAs) were used to measure T_4 and T_3 using the procedures outlined in Boggs et al. (Boggs et al., 2011). Briefly, a proximity-scintillation 96-well format RIA was run with standard curves and inter-assay variance samples on every plate. Plasma was diluted with phosphate buffered saline with gelatin for T_4 (1:20) and T_3 (1:15) measurements.

2.5 Statistics

Statistics were performed using JMP 10 statistical software (SAS Institute Inc., Cary, North Carolina) or R (R Core Team, Vienna, Austria) (R Core Team, 2014). When size was considered a significant factor, male and female alligator populations were split into different size classes. These categories were defined as juveniles (snout-vent length [SVL] < 100 cm, $n = 29$), small adults (100 cm < SVL < 135 cm, $n = 50$), and large adults (SVL > 135 cm, $n = 143$). These size classes were determined by previous studies on the reproductive hormone cycles of *A. mississippiensis* which illustrated differences in steroid hormones according to size (Hamlin et al., 2011; Hamlin et al., 2014).

For UI measurements, the uncertainties were combined according to ISO guidelines. (JCGM, 2008) The relative expanded uncertainty was then used to calculate absolute expanded uncertainties for each individual sample. The expanded uncertainty was computed as follows:

$$U_c = k[A_1^2 + A_2^2 + B_1^2 + B_2^2]^{1/2}$$

where U_c is the combined expanded uncertainty, A_1 is the replication uncertainty due to sample measurement replication, A_2 is the uncertainty due to the blank corrections, B_1 is the uncertainty due to the weighing, B_2 is the uncertainty due to the primary calibrant, and k is the coverage factor (2.5).

UI measurements did not conform to normality. Therefore, nonparametric statistics and a Bonferroni correction for post hoc multiple t-tests were conducted. Sex and SVL, a known cofactor for many thyroid related endpoints in alligators, (Boggs et al., 2011) were tested for significance in all analyses. Analysis of seasonal UI concentrations were conducted without large adult females or samples in January due to low sample sizes ($n = 4$ and 1 respectively). For thyroid hormone and UI correlations, the nonparametric Spearman's rho was used with $\alpha = 0.05$ considered significant. Seasonal analysis of UI was tested by rank transformed ANCOVA analyses (Iman, 1982) with SVL as a cofactor and a post hoc Bonferroni corrected Wilcoxon each pair test (significant $\alpha < 0.0009$) was conducted to detect significant differences between groups.

Water temperatures were analyzed for correlations to the thyroid hormones and to urinary iodine using Spearman's rho with $\alpha = 0.05$ considered significant. This analysis was to determine whether thyroid hormones or urinary iodine were correlated to seasonal changes in temperatures.

To test for correlations between SVL and alligator $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values we performed Pearson's test with $\alpha = 0.05$ considered significant. In addition, we performed a MANOVA analysis to test for significant effects of capture year, sex, and size class on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. For isotope mixing model analyses we used a Bayesian mixing model within the 'siar' package (version 4.2, of R; hereafter SIAR) (Parnell and Jackson, 2013) to estimate the proportion of prey resources contributing to the diet of individual *A. mississippiensis*. We used discrimination factors determined for *A. mississippiensis* keratinized scute tissue by Rosenblatt and Heithaus (2013):

$$\Delta^{13}\text{C}_{\text{tissue-diet}} = 0.61\text{‰} \pm 0.1212\text{‰} (\text{mean} \pm \text{SD})$$

$$\Delta^{15}\text{N}_{\text{tissue-diet}} = 1.22\text{‰} \pm 0.08\text{‰} (\text{mean} \pm \text{SD})$$

We grouped prey into freshwater/terrestrial and marine/estuarine categories, calculating end member means and standard deviations from prey isotope values measured from each habitat. Prey species used in end member calculations were selected based on previous diet studies of coastal alligator populations (Nifong et al., 2015; Nifong et al., 2012; Rosenblatt et al., 2015). The posterior distributions produced by SIAR provide plausible solutions for the proportional contribution of prey sources to the diet of a consumer and incorporates variation in consumer and prey isotope values as well as variation in discrimination factors to provide robust estimates of dietary patterns.

For thyroid hormone analyses, samples that were above or below the RIA standard curve (T_4 : $< 0.156 \text{ ng/mL}$ (1 sample) or $> 80 \text{ ng/mL}$ (42 samples, four of which were in the thousands); T_3 : $< 0.0585 \text{ ng/mL}$ (none required removal) or $> 30 \text{ ng/mL}$ (one sample equal to 178 ng/mL) were removed from statistical analyses as these values were inaccurate. All thyroid hormone analyses were checked for normality by the Shapiro-Wilk W Goodness-of-fit test and for equal variances by the Brown-Forsythe test. Concentrations of T_4 were log transformed and T_3 concentrations were square root transformed to meet normality. Sexual dimorphism and differences among size groups for T_4 and T_3 concentrations were tested using a t-test or ANOVA. Given the small sample size of large adult females ($n = 7$), this demographic was excluded from the remaining analyses. Juveniles were excluded from the seasonal analysis due to small sample sizes. Therefore seasonal analysis was conducted on all small adults and large adult males. Individual t-tests were performed by month to detect differences between large adult males and small adults.

3. Results

3.1 Urinary Iodine and Thyroid Hormones

The NIST certified mass fraction values for the control material, SRM 3668, with its expanded uncertainties were (140.7 ± 4.2) $\mu\text{g/L}$ for level 1 and (272.9 ± 12.0) $\mu\text{g/L}$ for level 2. The percent error for level 1 and level 2 were -1.4% and 1.3% respectively. The mass fraction results obtained in this study for the control material are in good agreement with the reference values for iodine.

UI concentrations were not significantly correlated with plasma thyroid hormone concentrations except for T_3 and UI concentrations in small adults. While a correlation between plasma T_3 and UI concentrations was detected when the population was analyzed as a whole ($p = 0.03$, $\rho = 0.18$), SVL was a significant correlate for both UI and T_3 ($p = 0.0003$, $\rho = 0.28$ and $p = 0.01$, $\rho = 0.10$; Figure 1). Therefore, size classes were analyzed individually. Small adults had a significant correlation between T_3 and UI ($p = 0.05$, $\rho = 0.24$) but no correlation between SVL and T_3 or SVL and UI. Juveniles displayed no significant correlations. Large adults maintained a correlation between SVL and UI ($p = 0.03$, $\rho = 0.24$) but did not have a significant correlation between T_3 and UI or SVL.

Analysis of all size groups and sexes demonstrated that SVL was significantly related to UI concentrations ($p = 0.0003$; $\rho = 0.28$; Figure 1). Therefore SVL was used as a cofactor for UI seasonal analyses or size classes were analyzed separately. Large adults had significantly higher UI concentrations compared to small adults ($\chi^2 = 0.01$), but sexual dimorphism was not significant within or among size classes (juveniles $\chi^2 = 0.16$, small adults $\chi^2 = 0.75$) (Figure 2A and 2B). Therefore, sexes were grouped together. Seasonal UI concentrations with SVL as a cofactor exhibited significant peaks in UI concentrations during May and April compared to a low in February (ANCOVA $p < 0.0001$; May > February $p = 0.0002$ and April > February $p = 0.0001$; Figure 2C). There were no significant differences in mean UI concentrations in the peak months among the years studied (2008 to 2010; April $\chi^2 = 0.72$ and May $\chi^2 = 0.66$). Urinary iodine was not significantly correlated with temperature.

3.2 Stable Isotope Analysis

A total of 314 alligators were captured from 2006 to 2013 and subject to stable isotope analysis of scute keratin. Body-size of individuals ranged from 40.4 cm to 184.5 cm SVL and sex ratio was male biased 2.4:1 (male: female). $\delta^{13}\text{C}$ values of alligators varied widely, ranging from -28.0‰ to -13.7‰ . $\delta^{15}\text{N}$ was less variable and ranged from 4.2‰ to 11.5‰ . Both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were positively correlated with SVL (Pearson's: $r(312) = 0.38$, $p < 0.001$ and $r(312) = 0.51$, $p < 0.001$, for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively) suggesting an increased reliance on marine/estuarine prey resources with increasing size. Isotope values of potential prey species from freshwater habitats were depleted in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ relative to marine/estuarine species (Figure 3, Table 1). This pattern was also evident in the end member values calculated for use in our mixing model analyses.

Prior to SIAR analyses, individuals were categorized into five sub-population groups outlined in the methods section based on significant effects of size class and sex on isotope values (MANOVA test: $F(2,308) = 44.49$, Wilk's $\Lambda = 0.78$, $p < 0.001$; $F(2, 308) = 4.25$,

Wilk's $\Lambda = 0.97$, $p = 0.01$; for size class and sex, respectively). Males were categorized into three sub-population groups: juveniles ($n = 29$), small adults ($n = 50$), and large adults ($n = 143$). Whereas females were separated into two sub-population groups: juveniles ($n = 11$) and adults ($n = 81$). We ran SIAR independently for each sub-population group. Each model simulation consisted of 500,000 iterations with a burn-in of 50,000 iterations and thin-by interval of 15.

For purposes of this study, we express results from SIAR model simulations in terms of the estimated proportion of marine/estuarine prey in the diet of each sub-population group. Median estimates for proportional contribution of marine/estuarine prey to diet ranged from 0.38 (95 % Bayesian Creditable Interval-BCI = 0.25–0.46) for adult females to 0.68 (95 % BCI = 0.64 to 0.71) for large adult males while juvenile females displayed more variable diets (median = 0.40, 95% BCI = 0.13 to 0.55; Figure 3, Table 2). Estimated contributions to the diet of all other size classes were intermediate between the extremes of adult females and large adult males. As indicated by the broader creditable intervals and greater standard deviations, our estimates of dietary contributions for juvenile females and small adult males were more variable as compared to all other groups.

When matched samples were available, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were tested for correlation between T_4 ($n = 53$), T_3 ($n = 33$), and urinary iodine ($n = 35$). Plasma T_4 and T_3 concentrations did not correlate with either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ (T_4 : $p = 0.32$ and 0.46 ; T_3 : $p = 0.83$ and 0.18). Urinary iodine was not significantly correlated to $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ ($p = 0.47$ and 0.10 respectively).

3.3 Seasonal Thyroid Hormones

Analysis of all size groups showed no significant difference in plasma T_3 or T_4 concentrations among the size groups or between sexes ($p = 0.40$ and 0.85 respectively). No sexual dimorphism was detected within any of the size groups for either of the thyroid hormones (T_4 : juveniles $p = 0.99$, small adults $p = 0.42$, large adults $p = 0.26$; T_3 : juveniles $p = 0.48$, small adult $p = 0.74$, large adults $p = 0.91$). Small adults and large male adults were analyzed for seasonal differences in thyroid hormones. There were no significant differences in seasonal plasma T_3 concentrations (small adults $p = 0.51$; large adults $p = 0.11$; Figure 4). Plasma T_3 concentrations were elevated for large adult males compared to small adults during the months of February and April ($t = 0.02$ and 0.04 , respectively). Plasma T_4 concentrations varied seasonally for small adults but not for large adults ($p < 0.0001$ and $p = 0.17$, respectively). Among the small adults, there were peaks in plasma T_4 concentrations during July and August and lows October through April, excluding February. Plasma T_4 concentrations were higher for large adult males than for small adults during the months of January and April ($t = 0.05$ and 0.05 , respectively).

Additional analyses looking at water temperature and thyroid hormone concentrations found that plasma T_4 was significantly positively correlated with water temperatures ($p < 0.0001$; $\rho = 0.299$) that were taken at the site of collection of the alligators during sampling. However, plasma T_3 was not correlated with water temperature.

4. Discussion and Conclusions

This study demonstrated that size of the alligator is a significant correlate for UI. The increase in UI with an increase in size is consistent with previous studies that indicate adult alligators allocate more foraging time in marine/estuarine environments than juvenile alligators, which could account for an increased excretion of UI (Nifong et al., 2014; Subalussy et al., 2009). Generally, large males had elevated UI, but there was no significant difference between the sexes when size was considered. While it is thought that females remain close to freshwater sources as they are ideal and required nesting sites, this difference in UI concentrations could be due to sexual dimorphic limitations on size. Previous studies show growth is greatly reduced once females attain approximately 260 cm total length (approximately 135 cm SVL) and males attain approximately 365 cm total length (approximately 190 cm SVL) (Woodward et al., 1995). Our data support this sexual dimorphism as only six females were above the 135 cm SVL size limit and labeled as a large adult. With insufficient data on large adult females to differentiate whether differences in UI are due to size or sex, both size and sex should be considered in future analyses. Unlike the small adults, among the large adults there is a lack of correlation between UI and T_3 concentrations. Plasma T_3 concentrations (regardless of season) were not significantly different among size classes but large adults had elevated UI excretion compared to the other size classes. A lack of correlation between UI and T_3 suggests that large adult alligators are able to regulate increased iodine intake through increased excretion to maintain steady plasma T_3 concentrations compared to their smaller counterparts.

Seasonal differences in UI could be related to temperature restriction on feeding as well as breeding activities. During February, temperatures may be too cold for alligators to meet the metabolic requirements necessary for feeding. Feeding in both estuarine and freshwater environments most likely decreases during these times due to a reduction in digestion and absorption (Coulson and Coulson, 1986). This could lead to low UI in February that is likely due to reduced feeding overall. During April and May at MINWR, ambient temperatures increase allowing the alligators, which are metabolically constrained poikilotherms, to increase foraging in the prey rich marine/estuarine habitats. However, water temperatures were not correlated with UI concentrations. This is most likely because UI is a short term analysis of marine feeding and might not yield a direct correlation to a temporal change such as seasonal temperatures. This does not eliminate the possibility of a seasonal influence on UI because body temperatures may be more closely correlated with a metabolic process such as UI excretion in an ectotherm. Body temperatures were not recorded for this study but merit further investigation. An alternative explanation for seasonal changes in UI could be reduced rainfall during April and May leading to drying or shrinking of ephemeral water bodies which could increase prey density. Increased feeding on estuarine prey trapped by receding water in marshes from reduced rainfall could increase UI. However, there were no significant differences in mean UI concentrations among the years studied despite a drought in May 2008 and a dramatic increase in rainfall in May 2009 (a difference of approximately 30 cm between the years) (Hamlin et al., 2014). Therefore, it is not likely that increased prey density due to drought is the cause of increased urinary iodine in this study. Alternatively, an increase in UI concentrations could be related to reproductive activities as April and May

are the courtship and breeding season for alligators in this region (Hamlin et al., 2011; Hamlin et al., 2014). There is anecdotal evidence for courtship activities occurring in the marine environments as males have been seen bellowing at MINWR in the marine environments. Potentially, males could be guarding territories and females could be feeding in the nutrient rich estuaries to stockpile nutrients for egg laying. However, all evidence is anecdotal and further studies exploring behavioral connections with mating season and consumption of estuarine prey would need to be explored.

Stable isotope analysis ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of scute keratin and prey resources enabled the assessment of long-term (≈ 1 year) dietary trends of American alligators inhabiting MINWR. Using isotopic mixing model analyses (SIAR) we found adult females likely consumed the least amount of marine/estuarine prey, whereas predictions for juvenile females were more variable. While predictions for marine/estuarine prey use for juvenile and small adult male sub-groups were slightly higher, these predictions were also highly variable. Alternatively, predictions for large adult males indicated a heavy reliance on marine/estuarine prey resources and this pattern was consistent among individuals as this sub-group had the least amount of variation. As an artifact of the SIAR model formulation, we are unable to discriminate whether the predicted proportional contributions represent the diet of the sub-group as a whole or the proportion of individuals predominately feeding on a specific resource. While the broad credible intervals of some groups represent some degree of individual variation, techniques to further explore the factors influencing this variation are beyond the scope of this study. Results from our mixing model analyses were consistent with short-term trends in prey resource use indicated by UI concentrations, suggesting a significant degree of dietary stability through time (at least over the duration of tissue turnover). Ontogenetic shifts in diet from freshwater to marine/estuarine prey have been described in other coastal American alligator populations (Nifong et al., 2015) and interestingly, adult females tend to forage in marine/estuarine habitats the least, most likely a result of reproductive behaviors (i.e., nesting and parental care). Furthermore, long-term dietary stability has recently been described in a number of American alligator populations, (Rosenblatt et al., 2015) and findings from this study provide additional evidence and indicate dietary stability is likely a common attribute of American alligator ecology.

The thyroid hormone profiles among the adult populations of alligators at MINWR varied seasonally or by size class during this study. Plasma T_4 concentrations varied seasonally for the small adults but not for the large adults. A peak in plasma T_4 concentrations in July and August was followed by depressed plasma concentrations from October to April in this study; this seasonal trend is seen in many reptiles and has been hypothesized to be related to temperature and/or photoperiod (Southwood et al., 2003; Virgilio et al., 2004). In this study there was a positive correlation between plasma T_4 and water temperatures which is consistent with previous studies on alligator thyroid hormones (Boggs et al., 2011). Although water temperatures were correlated with plasma T_4 concentrations, seasonal variation in plasma T_4 was not seen in the large adults. It has been suggested that body size could be a factor influencing thermoregulation through behavior and thermal inertia (Seebacher et al., 1999). Larger reptiles display a decrease in daily fluctuations of body temperature as well as an increase in the average body temperature, and plasma T_4 concentrations among reptiles are positively correlated with temperature (Southwood et al.,

2003; Virgilio et al., 2004). It therefore follows that stabilization of body temperature could explain the lack of variation in T_4 among the largest size class, as well as why larger adults had higher T_4 concentrations in January compared to smaller adults. Body temperatures were not collected in this study and may not be reliably correlated to water temperatures of where the animal was caught given the mobility of alligators to move across land and other water bodies. A correlation between body temperatures and thyroid hormones in large adults requires additional investigation.

Plasma concentrations of T_3 did not exhibit seasonal variation among size classes in this study. This differs from previous studies of this population as well as other reptiles, which describe T_4 and T_3 concentrations that fluctuate together, whereas in this study T_3 and T_4 concentrations had independent patterns. (Boggs et al., 2011; El-Deib, 2005; Sciarrillo et al., 2000; Sengupta et al., 2004; Virgilio et al., 2004). Thermal regulation could account for the lack of seasonality in T_3 concentrations among the larger adults as large adults had significantly higher T_3 concentrations when compared to small adults in February. However, the smaller adults also lacked seasonality of plasma T_3 concentrations. Potentially, T_3 concentrations could be consistently elevated by the availability of iodine in marine/estuarine prey, as isotope analyses demonstrated that all size classes and sexes showed some degree of reliance on marine/estuarine prey resources. Indeed, small adults, which include the breeding female population, have a significant increase in circulating T_3 concentrations that positively correlates with UI concentrations. Boggs et al. (2011) observed that juvenile alligators from MINWR had elevated plasma T_3 concentrations when compared to a freshwater population. The concentrations in the current study are similar to the range of plasma T_3 concentrations reported previously for MINWR (Boggs et al., 2011). This repeated pattern in combination with the significant correlation between plasma T_3 and UI concentrations lead us to hypothesize that small adults from MINWR could have elevated plasma T_3 concentrations due to increased marine/estuarine foraging. The small adult demographic contains the breeding female population of MINWR which suggests a possible influence of elevated maternal T_3 concentrations on the health of neonates. A previous study demonstrated that neonatal alligators from MINWR had plasma inorganic iodide concentrations that were highly correlated with elevated plasma T_3 concentrations compared to freshwater neonatal counterparts that lacked such correlation (Boggs et al., 2013). This previous study concluded that MINWR neonates demonstrated thyrotoxicosis due to an overproduction of T_3 which was likely due to a maternal over-contribution of iodine or thyroid hormones to the egg for development of the embryo. This study adds to the evidence that the health of the MINWR neonatal population could be affected by elevated maternal iodine. A future study is needed to examine this possibility in more detail.

UI concentrations provide “snapshot” information on thyroid health of a population, while stable isotope analysis gives a more concrete image of yearly dietary patterns and habitat use. However, both techniques clearly demonstrate that adult alligators in coastal environments are estuarine/marine foragers, which influences their thyroid hormone concentrations. Increased marine foraging described through increased UI and a highly marine isotopic signature among the large male population may explain why large males did not have seasonal differences in plasma T_4 concentrations. Therefore, large male alligators in coastal environments could have consistent plasma T_4 concentrations throughout the year

due to the availability of iodine in their diet. These consistent thyroid hormone concentrations may affect metabolism and behavior of this coastal population but further analyses comparing this population to a freshwater population is required to determine physiological effects due to the changes in thyroid hormones. Additionally this study describes a correlation between elevated plasma T₃ concentrations and increased UI excretion among the female breeding population, which could be further evidence of maternal over-contribution of iodine leading to neonatal thyrotoxicosis. The detailed information on the influence of ontogenetic niche shifts on thyroid hormone concentrations provided by this research can aid in the conservation of this top predator species.

Acknowledgments

We extend our thanks to the staff at InnoMedic Health Applications at KSC and the Florida Fish and Wildlife Commission for assistance in collection of the samples. We also thank the National Research Council Postdoctoral Fellowship (ASPB) as well as the Estuarine Reserves Division, Office of Ocean and Coastal Resource Management (JCN), National Ocean Service, National Oceanic and Atmospheric Administration (award #NA10NOS4200022; JCN), CoEE Center for Marine Genomics (LJG) and grants from NIST (LJG) and NASA (LJG) for funding this project in part.

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Highlights

- American alligators that feed in marine systems have increased urinary iodine
- Isotopic signatures showed large males primarily ate marine/estuarine prey
- Large adult males had elevated urinary iodine not correlated to thyroid hormones
- Small alligators had correlative increases in triiodothyronine with urinary iodine
- Niche shifts in feeding can alter iodine intake and thyroid hormones

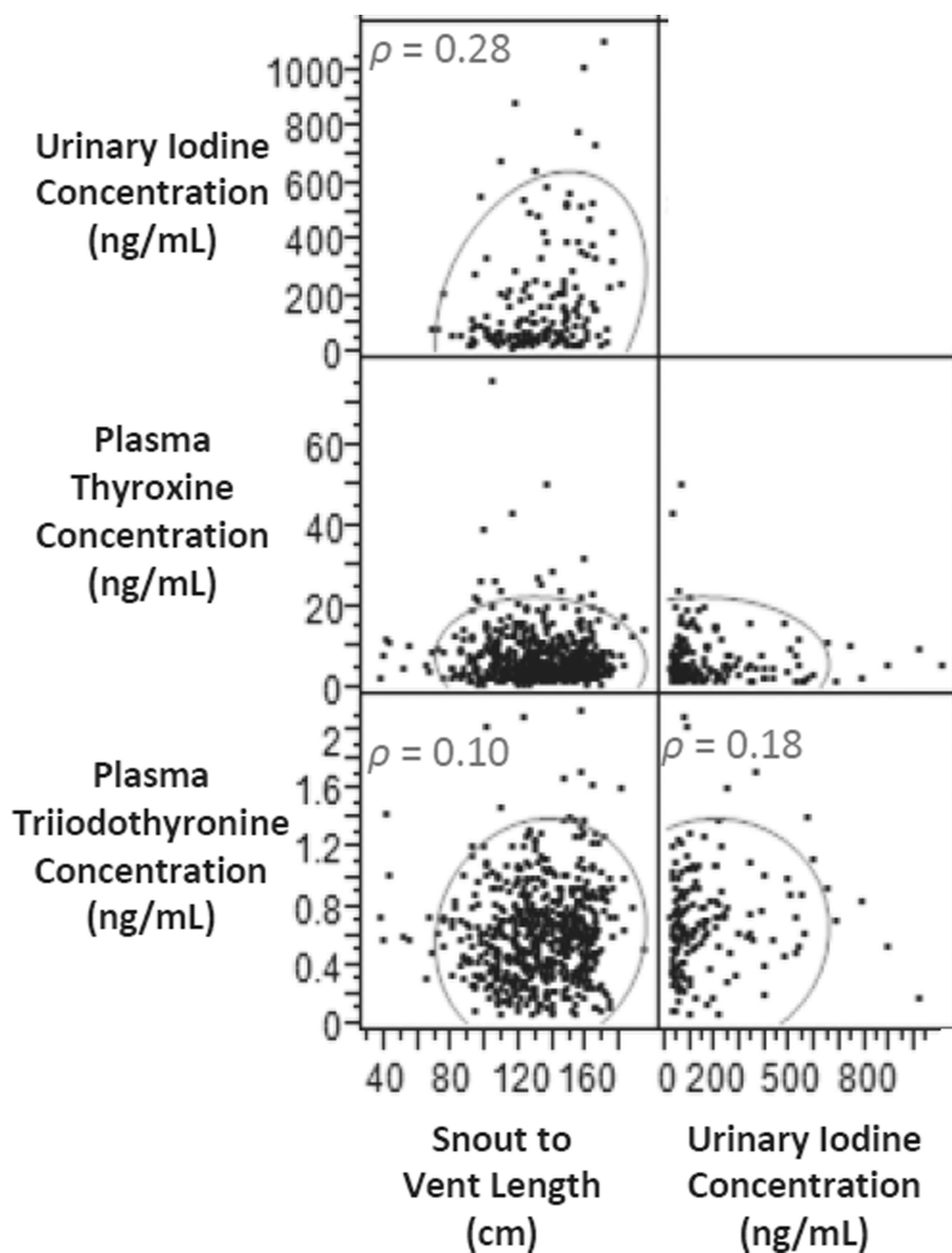


Figure 1. Correlations among urinary iodine UI, plasma thyroxine (T_4), plasma triiodothyronine (T_3), and body size (SVL) in *A. mississippiensis*. Rho (ρ) values are displayed for statistically significant relationships.

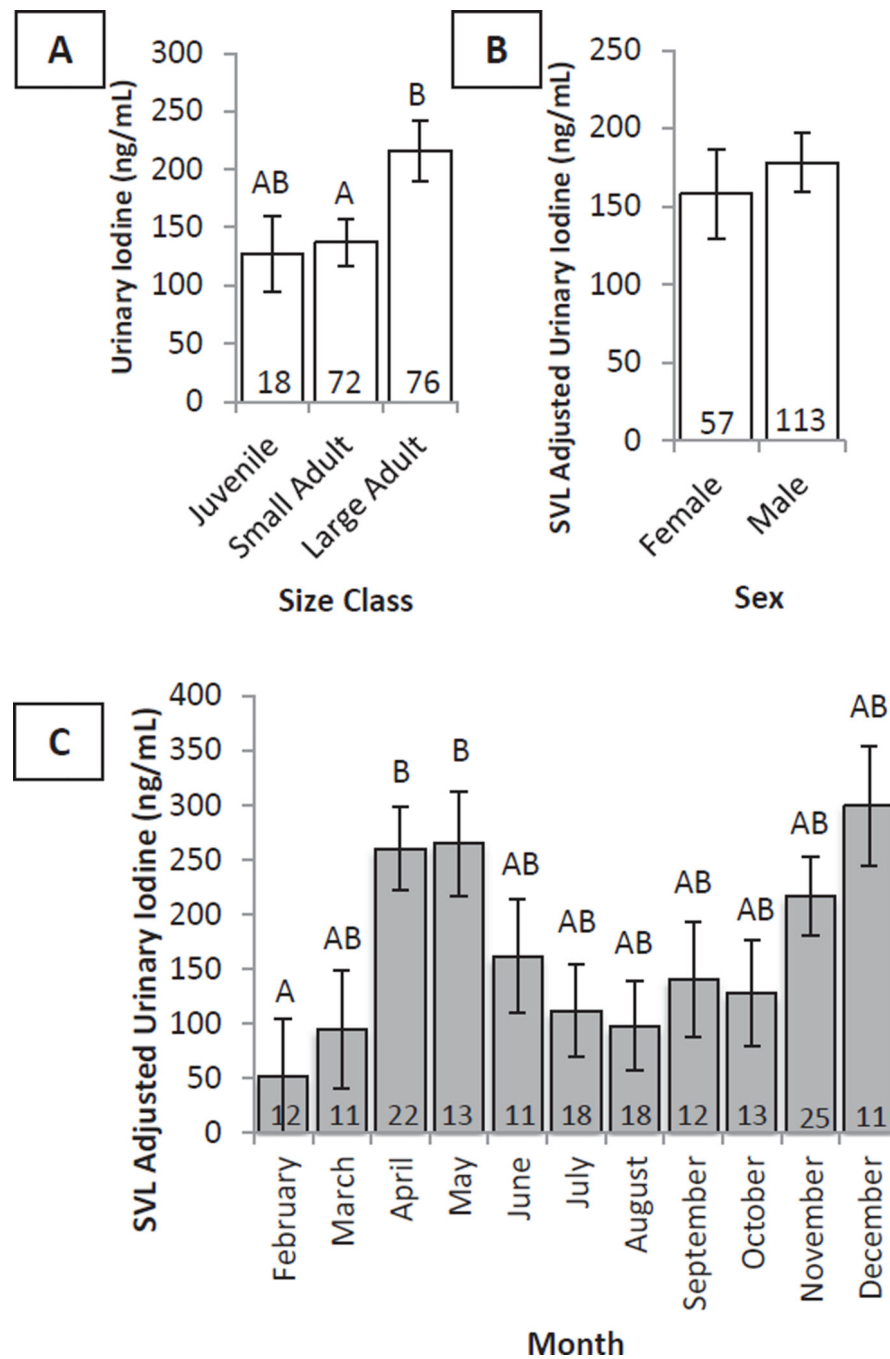


Figure 2. Demographics and seasonal patterns of urinary iodine concentrations in *A. mississippiensis*. Letters represent significant differences and numbers within the bars represent sample size. Error bars are \pm standard error. A. Age class differences in urinary iodine (UI) B. UI by sex. C. Unranked seasonal UI concentrations with snout to vent length (SVL) as a covariate. Letters represent significant differences and numbers within the bars represent sample size. Error bars are \pm standard error.

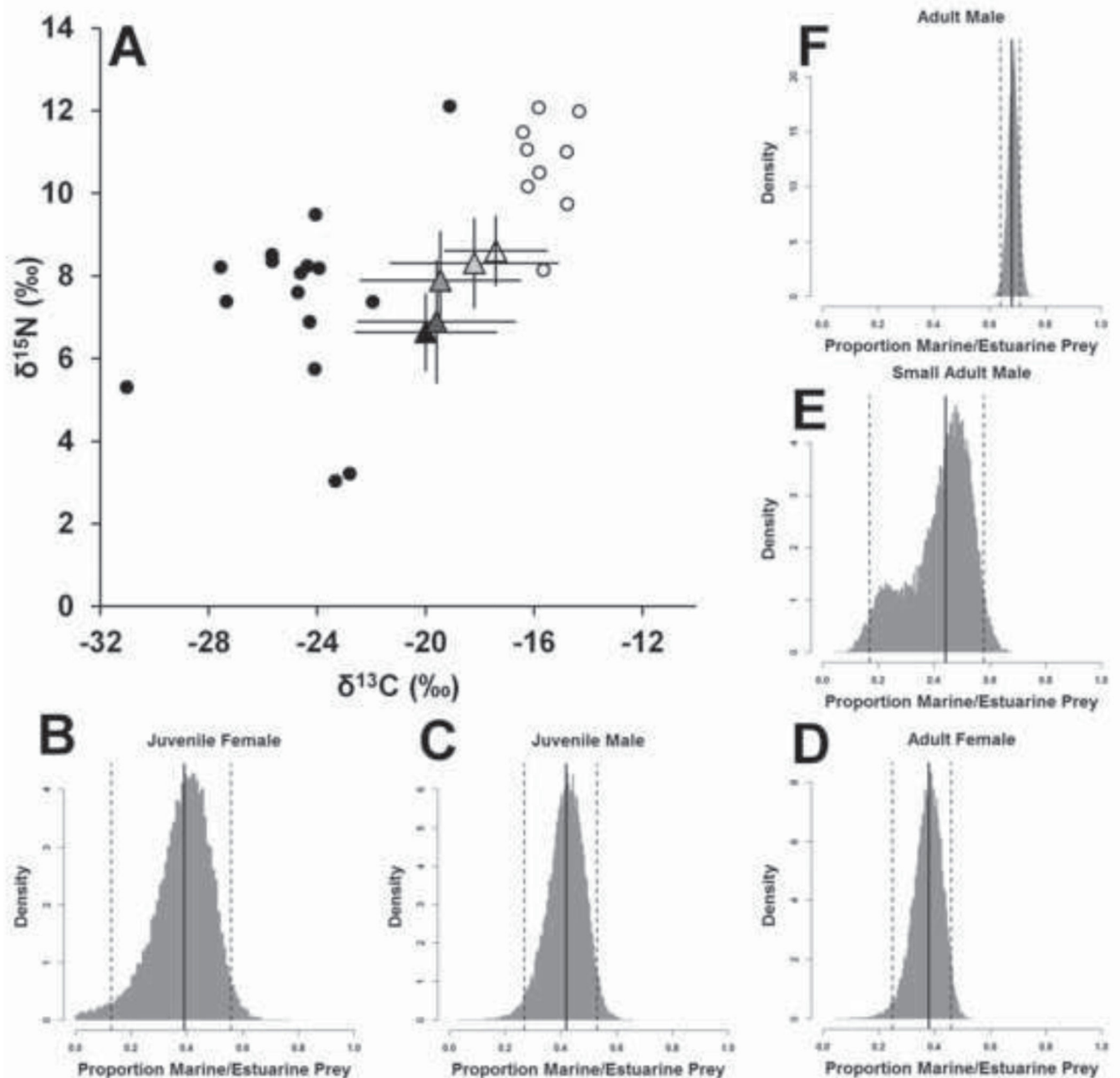


Figure 3.

Stable isotope analysis of *A. mississippiensis*. A) Mean stable isotope values ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) measured in American alligator sub-population groups (filled triangles: juvenile females-black, juvenile males-dark grey (25%), small adult males-light grey (75%), adult females-medium grey (50%), adult males-white) and potential prey species (filled circles: freshwater-black, marine/estuarine-white). Error bars are \pm standard deviation. Error bars are absent for prey species means for visual purposes. Panels B–F: Histograms represent the posterior probability distributions for the estimated proportional contributions of marine/estuarine prey to the diet of American alligator sub-population groups resulting from

isotopic mixing model analysis (SIAR). The solid lines are the median values and dashed lines constrain the 95% Bayesian Creditable Intervals (95% BCI).

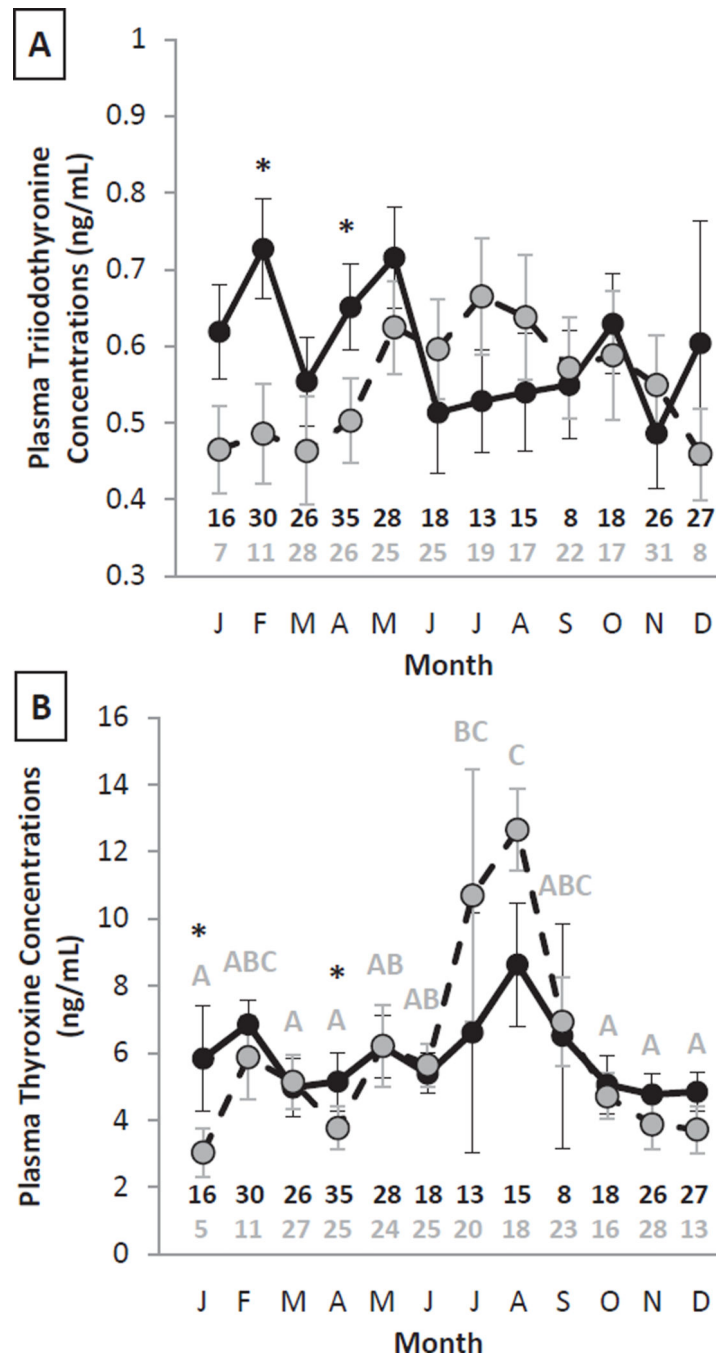


Figure 4.

Seasonal plasma thyroid hormone concentrations of *A. mississippiensis* at Merritt Island National Wildlife refuge. Grey circles and numbers and dotted lines represents small adults (snout-vent length [SVL] ≤ 135 cm) whereas black circles and numbers and solid lines represents large adults (SVL > 135 cm). Numbers under points represent sample size for that month (n). Significant differences among months are represented by letters. Significant difference between the two size classes by month are represented by an asterisk. Error bars

are \pm standard error. A. Plasma triiodothyronine (T_3) concentrations. B. Plasma thyroxine (T_4) concentrations.

Table 1

Stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) means, standard deviations, and number of samples (n) measured from potential prey species collected within Merritt Island National Wildlife Refuge during 2013.

Species (common name)	n	$\delta^{13}\text{C}$ (‰)		$\delta^{15}\text{N}$ (‰)	
		Mean	SD	Mean	SD
Marine/Estuarine Prey					
<i>Alpheus heterochaelis</i> (Bigclaw Snapping Shrimp)	2	-16.2	0.2	10.2	0.1
<i>Panopeus herbstii</i> (Blackclaw Mudcrab)	4	-15.8	0.5	10.5	0.3
<i>Arius felis</i> (Hardhead Catfish)	5	-16.4	0.7	11.5	0.6
<i>Elops saurus</i> (Ladyfish)	5	-15.8	0.9	12.1	0.5
<i>Fundulus similus</i> (Long-nosed Killifish)	1	-14.3	-	12.0	-
<i>Dasyatis sabina</i> (Atlantic Stingray)	9	-14.8	1.2	11.0	0.7
<i>Lagodon rhomboidalis</i> (Pinfish)	7	-16.2	1.4	11.1	0.4
<i>Mugil cephalus</i> (Striped Mullet)	7	-14.8	1.8	9.7	0.6
<i>Mugil curema</i> (White Mullet)	4	-15.6	0.4	8.1	1.5
End Member Value	-	-15.5	1.3	10.7	1.2
Freshwater Prey					
<i>Planorbidae</i> (Ramshorn Snail)	1	-23.3	-	3.0	-
<i>Palaemonetes</i> sp. (Grass Shrimp)	5	-24.7	1.5	7.6	1.8
<i>Belostoma</i> sp. (Giant Water Bug)	1	-22.8	-	3.2	-
<i>Fundulus chrysotus</i> (Golden Topminnow)	1	-23.9	-	8.2	-
<i>Fundulus seminolis</i> (Seminole Killifish)	1	-27.6	-	8.2	-
<i>Gambusia affinis</i> (Mosquitofish)	4	-24.4	1.2	8.2	1.9
<i>Lucania goodei</i> (Bluefin killiefish)	1	-24.3	-	6.9	-
<i>Lepisosteus platyrhincus</i> (Florida Gar)	2	-25.6	0.3	8.5	0.1
<i>Lepomis macrochirus</i> (Bluegill)	6	-24.6	1.2	8.1	0.4
<i>Lepomis marginatus</i> (Dollar Sunfish)	3	-27.3	1.5	7.4	0.2
<i>Lepomis microlophus</i> (Redear Sunfish)	2	-25.6	0.6	8.3	0.1
<i>Micropterus salmoides</i> (Largemouth Bass)	1	-24.1	-	9.5	-
<i>Mugil cephalus</i> (Striped Mullet)	2	-31.0	0.9	5.3	0.0

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Species (common name)	n	$\delta^{13}\text{C}$ (‰)		$\delta^{15}\text{N}$ (‰)	
		Mean	SD	Mean	SD
<i>Mugil curema</i> (White Mullet)	2	-21.9	0.7	7.4	0.4
<i>Poecilia latipinna</i> (Sailfin mollie)	2	-24.1	1.4	5.7	1.5
<i>Phalacrocorax auritus</i> (Cormorant)	1	-19.1	-	12.1	-
End Member Value	-	-24.9	2.4	7.5	1.8

Table 2

Number of individuals (n), stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) means and standard deviations, and estimated proportional contribution of marine/estuarine prey to diet of *Alligator mississippiensis* sub-population groups.

Group	n	$\delta^{13}\text{C}$ (‰)		$\delta^{15}\text{N}$		SD	Median	SD	95 % BCI
		Mean	SD	Mean	SD				
Juvenile female	11	-20.0	2.6	6.6	0.9	0.10	0.40	0.10	0.13 to 0.55
Juvenile male	29	-19.6	2.9	6.9	1.5	0.07	0.42	0.07	0.27 to 0.54
Small adult male	50	-18.2	3.1	8.3	1.1	0.11	0.45	0.11	0.17 to 0.58
Adult female	81	-19.5	2.9	7.9	1.2	0.38	0.05	0.05	0.25 to 0.46
Large adult male	143	-17.4	1.9	8.6	0.8	0.02	0.68	0.02	0.64 to 0.71